Plant Physiol. (1993) 103: 1009–1014

Current Nitrogen Fixation Is Involved in the Regulation of Nitrogenase Activity in White Clover (*Trifolium repens* L.)[1]

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Previous studies have shown that nitrogenase activity decreases dramatically after defoliation, presumably because of an increase in the O_2 diffusion resistance in the infected nodules. It is not known how this O_2 diffusion resistance is regulated. The aim of this study was to test the hypothesis that current N_2 fixation (ongoing flux of N_2 through nitrogenase) is involved in the regulation of nitrogenase activity in white clover (*Trifolium repens* L. cv Ladino) nodules. We compared the nitrogenase activity of plants that were prevented from fixing N_2 (by continuous exposure of their nodulated root system to an Ar:O_2 [80:20] atmosphere) with that of plants allowed to fix N_2 (those exposed to N_2:O_2, 80:20). Nitrogenase activity was determined as the amount of H_2 evolved under Ar:O_2. An open flow system was used. In experiment I, 6 h after complete defoliation and the continuous prevention of N_2 fixation, nitrogenase activity was higher by a factor of 2 compared with that in plants allowed to fix N_2 after leaf removal. This higher nitrogenase activity was associated with a lower O_2 limitation (measured as the partial pressure of O_2 required for highest nitrogenase activity). In experiment II, the nitrogenase activity of plants prevented from fixing N_2 for 2 h before leaf removal showed no response to defoliation. The extent to which nitrogenase activity responded to defoliation was different in plants allowed to fix N_2 and those that were prevented from doing so in both experiments. This leads to the conclusion that current N_2 fixation is directly involved in the regulation of nitrogenase activity. It is suggested that an N feedback mechanism triggers such a response as a result of the loss of the plant's N sink strength after defoliation. This concept offers an alternative to other hypotheses (e.g. interruption of current photosynthesis, carbohydrate deprivation) that have been proposed to explain the immediate decrease in nitrogenase activity after defoliation.

Various environmental stresses result in a rapid decrease in nitrogenase activity. In white clover (*Trifolium repens* L.), a widespread forage legume of temperate climates, defoliation is a frequently experienced stress and leads to a sharp decrease in nitrogenase activity within a few hours (Moustafa et al., 1969). Partial or complete recovery of nitrogenase activity following increases in pO_2 around the nodules led to the conclusion that the decrease in nitrogenase activity after leaf removal is due to an increase in nodule O_2 diffusion resistance rather than a lack of carbohydrate (Hartwig et al., 1987, 1990). However, it is not yet known how the change in O_2 diffusion resistance is regulated.

From the plant's responses to manipulations of either its N sink or its symbiotic N_2-fixing system (N source), it seems that the plant's demand for symbiotically fixed N is involved in the regulation of N_2 fixation. For example, altering the N source capacity by supplying nitrate to an N_2-fixing wild type and to a nitrate reductase-minus mutant of pea (Jacobsen, 1984; Shelp et al., 1991) resulted in a decrease in nitrogenase activity in the wild type but not in the mutant. The mineral N could not act as an additional N source for the mutant, and the plant remained solely dependent on fixed N to satisfy its N demands.

Kessler et al. (1990) attempted to affect the N source capacity by reducing root temperatures while leaving shoot temperature unchanged. No decrease in nitrogenase activity could be observed. This finding indicates that the unchanged shoot growth maintained the demand for N. Similarly, 5 d after faba beans had been deprived of 50% of their nodules, nitrogenase activity per plant was again at the level recorded before nodule removal, due to an increasing specific nitrogenase activity (Herdina and Silsbury, 1990). It seems that nitrogenase activity was adjusted to the plant N sink strength.

The responses to manipulations of the N sink capacity further support the hypothesis that N_2 fixation is influenced by the demand for symbiotically fixed N: The reduction of nitrogenase activity following pod removal or defoliation in soybean has been attributed to a corresponding reduced requirement for N (Fujita et al., 1988, 1991a). This may also be true after defoliation in white clover (Boller and Nösberger, 1988). In reciprocally grafted soybeans with different growth rates, total nitrogenase activity was associated with the shoot growth rate (Fujita et al., 1991b).

The objective of this study was to elucidate the possible relationships between N sink strength and N_2-fixing activity. Therefore, it seemed pertinent to examine whether current N_2 fixation (ongoing flux of N_2 through the nitrogenase) is involved in the regulation of nitrogenase activity. To prevent plants from fixing N_2, we exposed the nodulated root system to an Ar:O_2 (80:20) atmosphere. Nitrogenase activity responded differently to defoliation when nodules were exposed to N_2:O_2 (80:20), in contrast to those exposed to Ar:O_2 (80:20).

[1] Supported by a grant from the Swiss Federal Institute of Technology, Zurich, and in part by the Swiss National Science Foundation.

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Abbreviation: pO_2, partial pressure of oxygen.

MATERIALS AND METHODS

Plant Material and Growth Conditions

White clover plants (*Trifolium repens* L. cv Ladino) were grown from stolon cuttings (cloned from a single genotype) in growth chambers (PGR-15 Conviron Instruments Co., Winnipeg, Canada) at 18/13°C day/night temperatures and 80% RH, in a 16-h photoperiod with a PPFD of 450 to 500 μmol of quanta PAR m^{-2} s^{-1}. Plants were established by transplanting stolon tips, including the first four to five internodes, into special 250-mL gas-tight sealable pots filled with silica sand (grain diameter 0.8–1.2 mm). Plants were watered with a nutrient solution similar to that used by Hammer et al. (1978). With the exception of the first 3 weeks, when 3.5 mM $(NH_4)_2SO_4$ was added, the solution was N free. Plants were inoculated with *Rhizobium trifolii*, strain RCR 5 (Hup^-, Rothamstead, UK), on d 10, 15, and 20 after planting. Experiments were carried out 6 to 8 weeks after planting, when plants had attained a dry weight between 3 and 4 g. For defoliation, all leaves above the size of 0.3, as defined by Carlson (1966), were cut off at the base of the petiole.

Gas Exchange System

Nitrogenase (EC 1.7.99.2) activity was measured as H_2 evolution in Ar:O_2 (80:20) using a gas exchange system similar to that described by Minchin et al. (1983). Mass flow controllers (FC-260–4S; Tylan GmbH, Eching, Germany) allowed accurate mixing of pO_2 in Ar of the input gas. The output gas passed through an H_2 analyzer (Morgan Scientific, Inc., Andover, MA) after drying in an ice trap (isopropanol cooled by solid CO_2).

Measurement of Nitrogenase Activity and Determination of O_2 Limitation of Nitrogenase Activity

Fifteen hours before the experiment was started, the pots were sealed, connected to the gas exchange system, and subsequently flushed with ambient air at a flow of approximately 100 mL min^{-1}. Daytime conditions were applied within this period. Ninety to 120 min before H_2 evolution measurements, the input gas was changed to an N_2:O_2 (80:20) mixture and the flow increased to 400 mL min^{-1}. When steady H_2 evolution was attained, the root atmosphere was changed to Ar:O_2 (80:20), and nitrogenase activity was determined as the peak H_2 evolution. Roots were exposed to Ar:O_2 for no longer than 6 min for peak H_2 evolution measurements and thereafter were reexposed to N_2:O_2. Consecutive peak measurements were made hourly to allow recovery from the Ar-induced decline. Nitrogenase activity was calculated on the basis of whole plant dry weight.

An increased O_2 diffusion resistance leads to a higher O_2 limitation of nitrogenase activity, which can be overcome by increasing the rhizospheric pO_2. To estimate O_2 limitation, the pO_2 required for highest nitrogenase activity was determined. For this purpose, the content of O_2 in Ar was stepwise increased from 20 to 40 to 60 to 90% (v/v) at 60-s intervals beginning 1 min after peak H_2 evolution under 20% O_2 (Fig. 1). Peak H_2 evolution values were fitted (Sigmaplot 4.1; Jandel Scientific, Corte Madera, CA). The equation obtained

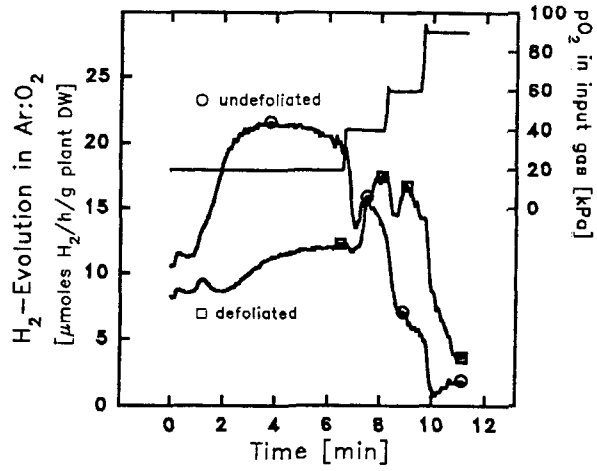

Figure 1. Determination of O_2 limitation of nitrogenase activity. Representative time course showing the response of H_2 evolution to stepwise increased pO_2 of defoliated and undefoliated plants. Symbols mark peak H_2 evolution at a given pO_2. See corresponding section in "Materials and Methods" for further explanation.

was used to calculate the pO_2 at which nitrogenase activity reached its highest value. This reflects the O_2 limitation of nitrogenase activity presumably caused by the variable O_2 diffusion resistance in the nodule. It was assumed that the O_2 diffusion resistance is able to increase within minutes. This was shown in various treatments (reviewed by Dakora and Atkins, 1989; Layzell and Hunt, 1990), including the response to Ar and to elevated rhizospheric pO_2 (King and Layzell, 1991). Therefore, the O_2 limitation of nitrogenase activity due to defoliation may be overestimated. To minimize this possible error, measurements of the response of nitrogenase activity to increased pO_2 were performed very rapidly over a wide range of O_2 concentrations.

NH_4^+ Analysis

Plants were frozen in liquid N_2 no later than 20 s after removal from the pots. The nodules were kept frozen while they were picked off the roots. Nodule material, after grinding in a mortar cooled with liquid N_2, was extracted with 1.125 M H_2SO_4 and centrifuged for 20 min at 19,000*g*. NH_4^+ concentrations were measured colorimetrically (spectrophotometer U-2000; Hitachi, Tokyo, Japan) after reaction with KOH-phenolate (Bohley, 1967). The detection limit was 1 μg of NH_4^+ ml^{-1} of sample solution.

Statistical Analyses

Data were analyzed with Statgraphics version 5.0 (Statistical Graphics Corp., Rockville, MD). Results are expressed as means ± SE of at least four replicates. Each experiment was conducted at least twice.

RESULTS

To investigate the influence of current N_2 fixation on the response of nitrogenase activity to defoliation, current fixa-

tion of N_2 was prevented in two different experiments. In experiment I, leaf removal was followed by continuous prevention of N_2 fixation for 6 h. In experiment II, conditions preventing N_2 fixation preceded defoliation for 2 h and continued thereafter for 3 h. At the same time tests were conducted to determine whether observed changes in nitrogenase activity after defoliation might be caused by changes in the O_2 limitation.

Nitrogenase Activity and O_2 Limitation when N_2 Fixation Was Prevented for 6 h after Defoliation

Before defoliation, the mean nitrogenase activity measured as peak H_2 evolution was 14.7 ± 1.4 μmol H_2 h^{-1} g^{-1} plant dry weight (=100%). To check the Ar-induced decline at the beginning of the experiment, nodules were exposed to Ar:O_2 for 20 min. During that time nitrogenase activity decreased to 65.2 ± 1.8% (Fig. 2). In experiment I, plants allowed to fix N_2 were exposed to Ar:O_2 only for 6 min every hour to determine nitrogenase activity values. Under this regime, the nitrogenase activity of undefoliated plants recovered from the initial Ar-induced decline during the 6-h experimental period; after 6 h they showed 97.0 ± 7.0% of the H_2 evolution as compared to the initial activity. In contrast, undefoliated plants prevented from fixing N_2 by exposure to Ar:O_2 showed a constant nitrogenase activity after the initial Ar-induced decline; after 6 h, nitrogenase activity was 59.4 ± 7.7% of the initial value.

The nitrogenase activity of defoliated plants allowed to fix N_2 between each 6-min measurement declined rapidly within 4 h of leaf removal. The activity 6 h after defoliation was 20.5 ± 2.0% of the peak activity before defoliation. By contrast, the nitrogenase activity of plants with nodulated roots kept continuously under Ar:O_2 after defoliation and never fixing N_2 during the 6-h experimental period did not decrease to the same extent as that of the defoliated plants allowed to fix N_2. In this case, the nitrogenase activity declined only to 37.4 ± 4.0% of peak activity before defoliation.

The severe decrease in nitrogenase activity in nodules of defoliated plants allowed to fix N_2 between measurements was associated with a stringent O_2 limitation of nitrogenase activity (Table I). In plants kept continuously under Ar:O_2, there was a lesser degree of O_2 limitation of nitrogenase activity, either in undefoliated or in defoliated plants.

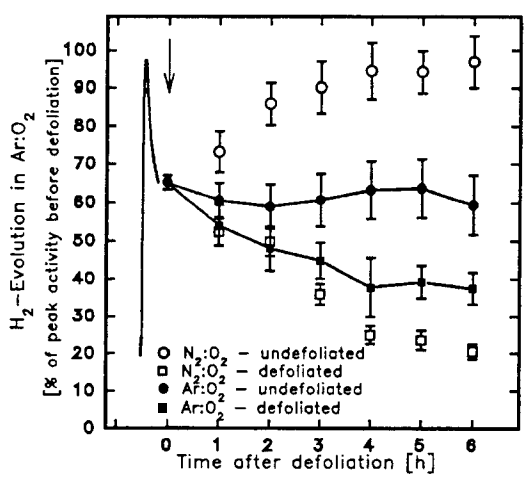

Figure 2. Response of nitrogenase activity (H_2 evolution in Ar:O_2 [80:20]) to defoliation followed by conditions preventing N_2 fixation. N_2 fixation was prevented by continuously replacing N_2 by Ar. N_2-fixing plants were exposed to Ar:O_2 for only 6 min hourly to determine peak H_2 evolution. Before defoliation, all plants were subjected to Ar:O_2 for 20 min to determine the pretreatment peak H_2 evolution and the severity of the Ar-induced decline. The arrow indicates the time of defoliation. Means ± SE of 16 or 4 replicates are shown (pre- or postdefoliation period, respectively).

Table I. *O_2 limitation (shown as pO_2 required for highest nitrogenase activity) 6 h after defoliation*

Nitrogenase activity was determined as H_2 evolution under Ar:O_2. The pO_2 was determined by increasing the rhizospheric pO_2 stepwise from 20 to 40 to 60 to 90% (v/v) (see corresponding section in "Materials and Methods" for further explanation). Plants were prevented from fixing N_2 by continuously exposing the roots to Ar:O_2 (80:20). Values represent means ± SE of four replicates. Data are from the experiment presented in Figure 2.

	pO_2 at Highest Nitrogenase Activity	
	Not defoliated	Defoliated
	kPa	
Allowed to fix N_2 (N_2:O_2)	≤40	70.7 ± 1.4
N_2 fixation prevented (Ar:O_2)	63.4 ± 1.5	62.3 ± 1.5

Nitrogenase Activity and O_2 Limitation when N_2 Fixation Was Prevented for 2 h before and for 3 h after Defoliation

Nitrogenase activity of plants exposed to Ar:O_2 for 2 h before defoliation showed no significant response to leaf removal during another 3 h under Ar:O_2 (Fig. 3). In contrast, the nitrogenase activity showed a significant decrease after

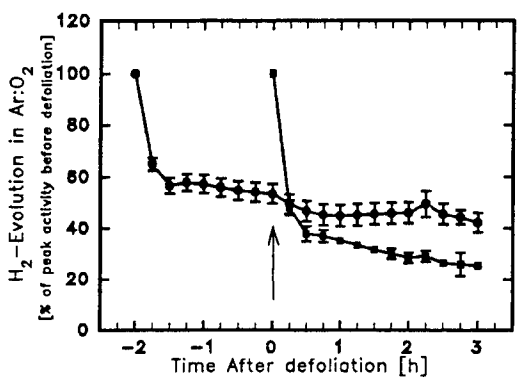

Figure 3. Response of nitrogenase activity (H_2 evolution in Ar:O_2 [80:20]) to leaf removal under conditions preventing N_2 fixation following defoliation after a 2-h pretreatment in either Ar:O_2 or N_2:O_2. Time course showing the response of H_2 evolution (mean ± SE) continuously measured under Ar:O_2; the arrow indicates the time of defoliation. ●, Plants exposed to Ar:O_2 for 2 h before defoliation (n = 18); ■, plants fixing N_2 before defoliation (n = 13).

defoliation if plants were exposed to Ar:O_2 for only 6 min to determine initial nitrogenase activity. Three hours after defoliation, the nitrogenase activity relative to initial peak activity (=100%) was 42.3 ± 3.8% in plants prevented from fixing N_2 before defoliation, compared to 25.3 ± 1.7% for plants allowed to fix N_2 before leaf removal.

The O_2 limitation determined 3 h after defoliation did not differ, despite the fact that the nitrogenase activity was different because of the two treatments. Plants defoliated after preventing N_2 fixation for 2 h attained their highest nitrogenase activity at 59.2 ± 0.6 kPa O_2. Plants allowed to fix N_2 before leaf removal attained a pO_2 for highest activity at 60.9 ± 1.2 kPa O_2.

Whole Nodule NH_4^+ Content after Defoliation

To detect whole nodule NH_4^+ concentration before and after defoliation, this primary N_2 fixation product was measured. Before defoliation, whole nodule content of NH_4^+ was 89 ± 9 ng of NH_4^+ mg^{-1} nodule dry weight (Table II). After defoliation, NH_4^+ content did not change significantly within 2 h. In another experiment, the effect of a 6-h Ar exposure on nodule NH_4^+ was determined. The NH_4^+ content in nodules kept under N_2:O_2 was 116 ± 42 ng mg^{-1} nodule dry weight; in nodules kept under Ar:O_2, it was 150 ± 50 ng mg^{-1} nodule dry weight. The results obtained in the present study are comparable to those for soybean (Streeter, 1987) and alfalfa nodules (Becana et al., 1985).

DISCUSSION

Involvement of Current N_2 Fixation in the Regulation of Nitrogenase Activity

The response of nitrogenase activity to defoliation in plants prevented from fixing N_2 was different from that in plants allowed to fix N_2: When N_2 fixation was prevented, the inhibition of nitrogenase activity due to defoliation was lower in experiment I (Fig. 2) and absent in experiment II (Fig. 3). This dependency of the response of nitrogenase activity to defoliation on the ability to fix N_2 allows us to conclude that current N_2 fixation is linked to the regulation of nitrogenase activity.

Ar, a chemically inert gas, was used frequently as a tool to switch off N_2 fixation, presumably without affecting whole plant metabolism. Atkins et al. (1984) exposed nodulated roots to Ar:O_2 for up to 3 d and observed no effect on nodule growth, on plant cell or bacteroid protein, or on nitrogenase activity. Zhu et al. (1991) observed increased dry weight and elevated nitrogenase activity in young soybean seedlings grown continuously in Ar:O_2 for 21 d. However, Ar does affect nodule metabolism: It causes the "Ar-induced decline" in nitrogenase activity (Witty et al., 1984; Hunt et al., 1987). This phenomenon is poorly understood; it is associated with a decrease in nodule permeability (Witty et al., 1984; Hunt et al., 1987) and tends to vary greatly. In undefoliated plants nitrogenase activity, due to the Ar-induced decline, was 40% lower than it was under N_2:O_2 (experiment I, Fig. 2). In contrast, in defoliated plants, nitrogenase activity was twice as great under Ar as compared to N_2:O_2. We can, therefore, assume that the Ar-induced decline did not slow down overall plant metabolism.

The decrease in nitrogenase activity after leaf removal has been ascribed to a decrease in the supply of O_2 to the infected zone of the nodule, presumably due to an increase in O_2 diffusion resistance in the nodules (Hartwig et al., 1987, 1990; Denison and Layzell, 1991; Denison et al., 1992; Diaz del Castillo et al., 1992). The present data confirm this suggestion (Table I). The greater decrease in nitrogenase activity due to defoliation under N_2:O_2 than under Ar:O_2 (Fig. 2) is associated with a greater O_2 limitation of nitrogenase activity (Table I). This presumed decrease in O_2 supply suggests that current N_2 fixation is involved in the regulation of the availability of O_2 for the bacteroid respiratory system.

In nodules exposed to Ar:O_2 a certain change in O_2 limitation occurs with the decrease in nitrogenase activity (Figs. 2 and 3; Table I). If such plants were defoliated, nitrogenase activity decreased further, but the O_2 limitation remained stable. The previous model, suggesting that a short-term response of nitrogenase activity is associated with a corresponding change in O_2 limitation, would not predict such behavior. It may be possible that the method used does not detect small differences in the O_2 limitation of nitrogenase activity. Purcell and Sinclair (1990) made observations consistent with ours by increasing rhizospheric partial pressure of NH_3, which induced an inhibition of nitrogenase activity. However, the inhibition was not concurrent with a reduction in nodule gas permeability, which decreased only after nitrogenase activity declined. Consequently, we must consider the possibility that another, unknown regulatory mechanism, in addition to a change in O_2 availability to the bacteroid respiratory system, is involved in the regulation of nitrogenase activity after defoliation.

Table II. *Whole nodule content of NH_4^+ after defoliation*

NH_4^+ content was determined colorimetrically. Values represent means ± SE of four replicates.

Time after Defoliation	NH_4^+ Content
min	*ng mg^{-1} nodule dry wt*
0	89 ± 9
15	83 ± 8
30	106 ± 18
60	90 ± 9
120	99 ± 6

What Triggers Short-Term Changes in Nitrogenase Activity in Nodules after Defoliation?

After defoliation, the shoot's N sink strength is reduced because of the loss of a significant part of the meristematic shoot. This has been shown by Boller and Nösberger (1988), who observed a significantly lower N uptake during the first days after defoliation. Leaf removal also reduces the xylem flow and may prevent the fixed N from being transported from the nodules to the shoot. Therefore, the inhibition of nitrogenase activity after defoliation might be the result of a feedback mechanism. Replacing N_2 by Ar after defoliation (Fig. 2, experiment I) prevented any further production of N compounds from current N_2 fixation, and as a result reduced

the possibility of an effective accumulation of N compounds. Consequently, the decrease in nitrogenase activity due to defoliation is significantly lower under these conditions than it is when N_2 is fixed. Similarly, the replacement of N_2 by Ar 2 h before leaf removal (experiment II) presumably drained the nodule of N_2 fixation products. Prolongation of the replacement of N_2 by Ar beyond defoliation makes an accumulation of N_2 fixation products very unlikely, which may explain the absence of a response of nitrogenase activity to defoliation (Fig. 3).

Several reports showing that nitrogenase activity was influenced by varying the O_2 diffusion resistance are consistent with the present observation that current N_2 fixation is involved in regulating nitrogenase activity. The defoliation and dehydration (Durand et al., 1987) of nodules both decrease nitrogenase activity. In each case, it can be assumed that the xylem flow is lowered. Thus, N_2 fixation products may not be transported from the nodules, and their accumulation induces a decrease in nitrogenase activity. The supply of nitrate, as an additional N source, inhibits nitrogenase activity (reviewed by Streeter, 1988). This may also be due to an accumulation of current N_2 fixation products (Streeter, 1985). Davey and Simpson (1989) and Hunt et al. (1989) increased the rhizospheric pO_2 and obtained a temporarily higher nitrogenase activity. This may have led to an inappropriately high N_2 fixation, because the N sink strength of the shoot was not affected by increasing rhizospheric pO_2. As a result, increased fixation of N_2 may have produced an accumulation of N products. This may have reduced nitrogenase activity to its initial level by increasing the O_2 diffusion resistance in the nodules.

Conversely, reducing the rhizospheric pO_2 to the suboptimal level of 10 kPa resulted in an initial decrease in nitrogenase activity (Weisz and Sinclair, 1987a, 1987b). The activity recovered completely 1 d later, presumably to meet the host's N demand. The steady increase of nitrogenase activity during regrowth after defoliation (Hartwig et al., 1987, 1990; Gordon et al., 1990) can be similarly explained. During regrowth, N_2 fixation is enhanced concurrently with the reconstitution of the shoot's N demand. In these two cases—after the reduction of rhizospheric pO_2 below 20 kPa and during regrowth—it appears that, because of the host's demand for fixed N, nodules are drained of products from N_2 fixation, which results in a lowering of the O_2 diffusion resistance and a consequent adjustment in nitrogenase activity. Therefore, we hypothesize that the balance between plant N sink strength and N_2 fixation products is maintained by adjusting the O_2 diffusion resistance and, thus, nitrogenase activity.

The regulation of nitrogenase activity by current N_2 fixation products could involve several N compounds. The primary fixation product is NH_4^+ (Kennedy, 1966) and, as a first step, it was tested as a possible regulating compound for nitrogenase activity. However, defoliation was observed to have no significant influence on the NH_4^+ content in a nodule crude extract (Table II). Similarly, Ohyama and Harper (1991) did not find any change in the amino or ureide N content in soybean nodules 2 h after decapitation, but they did report a significant increase of these compounds in the roots. Obviously, the ammonia concentration in the nodules is fairly constant, because a 6-h Ar exposure did not affect the nodule NH_4^+ content. This is consistent with the present result in which ammonia in the nodules was not identified as a trigger for nitrogenase activity.

Oti-Boateng and Silsbury (1993) proposed a feedback control mechanism to explain the inhibition of N_2 fixation by combined N. They suggested that amino acids are involved in such a mechanism. Parsons et al. (1993), after observing increased nodulation under $Ar:N_2:O_2$ compared to $N_2:O_2$, even proposed that symbiotic N fixation in general is regulated by N feedback mechanisms. Their suggestion coincided with a model of nodule metabolism that proposed that an N_2 assimilation sensor is connected to the regulation of nitrogenase activity (Layzell and Hunt, 1990). We suggest that this sensor detects either the accumulation or the lack of N_2 fixation products. This subsequently triggers an increase or decrease, respectively, in the O_2 diffusion resistance, thereby regulating nitrogenase activity. Such a mechanism would have to be directly related to the plant's N demand. Alternatively, it would allow the host to detect a failure in N_2 fixation by the bacteroids and enable the plant to avoid a loss of control over its energy-consuming symbionts. This may in part explain the Ar-induced decline, which cannot be explained by the hypothesis offered in the present study.

Our results are consistent with the hypothesis that the demand for symbiotically fixed N is an important element in the regulation of nitrogenase activity. We propose that an N feedback mechanism is involved in the regulation of nitrogenase activity after defoliation. This would provide an alternative explanation to other hypotheses (mainly, carbohydrate deprivation and the interruption of current photosynthesis), which have not been proven.

ACKNOWLEDGMENTS

We thank Prof. Dr. U. Feller (University of Berne, Switzerland) for his help with the NH_4^+ analysis and Dr. J.-J. Drevon (Institut National de la Recherche Agronomique, Montpellier, France) for helpful discussions.

Received February 9, 1993; accepted July 24, 1993.

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